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Effects of K074 and pralidoxime on antioxidant and acetylcholinesterase response in malathion-poisoned mice

Alessandra Antunes dos Santos [a,*], Danúbia Bonfanti dos Santos [a], Renata Pietsch Ribeiro [a], Dirleise Colle [a], Kaite Cristiane Peres [a], Júlia Hermes [a], Anderson Machado Barbosa [a], Alcir Luiz Dafré [a], Andreza Fabro de Bem [a], Kamil Kuca [b,c], Marcelo Farina [a]

[a] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040900, Florianópolis, Santa Catarina, Brazil
[b] Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Hradec Kralove, Czech Republic
[c] University Hospital, Hradec Kralove, Czech Republic

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ABSTRACT

The organophosphorus (OP) pesticide malathion is a highly neurotoxic compound and its toxicity is primarily caused by the inhibition of acetylcholinesterase (AChE), leading to cholinergic syndrome. Although oximes have been used as potential antidotal treatments in malathion poisoning because of their potential capability to reactivate the inhibited enzyme, the clinical experience with the clinically available oximes (e.g. pralidoxime) is disappointing and their routine use has been questioned. In the present study, we investigated the potency of pralidoxime and K074 in reactivating AChE after acute exposure to malathion, as well as in preventing malathion-induced changes in oxidative-stress related parameters in mice. Malathion (1.25 g/kg, s.c.) induced a significant decrease in cortico-cerebral, hippocampal and blood AChE activities at 24 h after exposure. Oxime treatments (1/4 of LD_{50}, i.m., 6 h after malathion poisoning) showed that pralidoxime significantly reversed malathion-induced blood AChE inhibition, although no significant effects were observed after K074 treatment. Interestingly, both oximes tested were unable to reactivate the cortico-cerebral and hippocampal enzymes after intramuscular or intracerebroventricular injection (1/4 of LD_{50}, 6 h after malathion poisoning). Biochemical parameters related to oxidative stress (cerebro-cortical and hippocampal glutathione peroxidase, glutathione reductase and catalase activities, as well as lipid peroxidation) were not affected in animals treated with malathion, oximes or atropine alone. However, pralidoxime and K074, administered intramuscularly 6 h after malathion poisoning, were able to increase the endogenous activities of these antioxidant enzymes in the prefrontal cortex and hippocampus. Taken together, the results presented herein showed that pralidoxime (the most common clinically used oxime) and the recently developed oxime K074, administered 6 h after malathion poisoning, were unable to reactivate the inhibited AChE in mouse prefrontal cortex and hippocampus. However, only pralidoxime significantly reversed the blood AChE inhibition induced by malathion poisoning. This indicates that peripheral and central AChE activities are not necessarily correlated after the treatment of OP compounds and/or oximes, which should be taken into account in the diagnosis and management of OP-exposed humans. In addition, considering that the available treatments to malathion poisoning appear to be ineffective, the present study reinforce the need to search for potential new AChE reactivators able to efficiently reactivate the brain and blood AChEs after malathion poisoning.

1. Introduction

Organophosphorus (OP) compounds display a broad variety of effects toward biological systems. From a toxicological point of view, they are well-known inhibitors of cholinesterases (ChEs), leading to the accumulation of acetylcholine in the synaptic terminals at the central and peripheral nervous systems, causing the consequent overstimulation of the cholinergic pathways (Bajgar, 2004; Bartling et al., 2007; Kwong, 2002).

The diagnosis of OP poisoning is based on anamnesis, the clinical status of the intoxicated organism, and on ChE determination in the blood (Bajgar, 2004). The measurement of this peripheral marker (activity of blood ChEs) has been extremely

* Corresponding author. Tel.: +55 4837219589; fax: +55 4837219672.
E-mail addresses: alessandraantunes@hotmail.com (A.A. dos Santos), farina@ccb.ufsc.br (M. Farina).

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useful to predict the extent of OP poisoning. In fact, there are many reports underlining the appropriateness of blood ChE as a surrogate parameter that mirrors the synaptic enzyme (Eyer et al., 2007).

Malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithioate) is an OP compound widely used in agriculture and veterinary practices as well as in attempts at suicide (Maroni et al., 2000). Of particular importance, our Toxicological Information Centre (Florianópolis, SC, Brazil) has reported that most of the cases of OP poisoning attended in our University Hospital were due to the exposure to malathion. Its toxicity requires the bioactivation to malaoxon (Buratti et al., 2006; Forsyth and Chambers, 1989), which is the molecule able to phosphorylate and inactivate ChEs (Taylor et al., 1995) – mainly acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Similarly to others OP compounds, malathion also leads to cholinergic syndrome (Abou-Donia, 2003; Brocardo et al., 2005; Franco et al., 2009; Trevisan et al., 2008).

Despite the clear involvement of AChE in the deleterious symptoms elicited by malathion, other targets and events potentially involved in its toxicity have been reported (Bajgar et al., 2007; Brocardo et al., 2005; Ranjbar et al., 2002, 2010; Videira et al., 2001). Particularly, oxidative damage produced by increased levels of lipid peroxidation (Akhgari et al., 2003; Ranjbar et al., 2010) and altered activities of antioxidant enzymes (Akhgari et al., 2003; Franco et al., 2009; Ranjbar et al., 2010; Trevisan et al., 2008) has been extensively reported. However, the exact mechanism by which malathion and others OP compounds induce oxidative damages is not fully understood.

The standard treatment for OP intoxication usually consists of the administration of a muscarinic cholinergic receptor antagonist (e.g. atropine) in combination with a reactivator containing an oxime functional group (da Silva et al., 2008; dos Santos et al., 2010). Anticholinergic drugs block the effects of overstimulation by accumulated acetylcholine at peripheral muscarinic receptors while the oximes, as compounds with nucleophilic activity, are able to break down the bond between the enzyme and the OPs inhibitors (dephosphorylation), releasing the enzyme to perform its physiological role (Bartosova et al., 2006; Kassa et al., 2007). Oximes have been investigated for many years as compounds with a great potential to the treatment of OPs poisoning (Dawson, 1994), however, the efficacy of commonly used oxime reactivators is still not sufficient and few compounds have found clinical application (Antonijevic and Stojiljkovic, 2007; Jokanovic and Stojiljkovic, 2006).

As the ability of the currently oximes used for the antidotal treatment of OPs poisoning (e.g. pralidoxime) is not fully satisfactory, the replacement of commonly used oximes by more effective molecules has been a long-standing goal (Kassa et al., 2007). Of particular interest, the antidotal treatment of malathion poisoning with pralidoxime (the standard oxime used in such condition) has not been efficient to reactivate inhibited AChE (Sudakin et al., 2000). In this scenario, there is a clear demand for new reactivators of malaoxon-inhibited AChE with a higher efficacy than those clinically available.

A recent study from our group showed that K074, a relatively new developed oxime that was synthesized at the Department of Toxicology, Faculty of Military Health Sciences, Hradec Kralove, Czech Republic, presented an outstanding reactivating effects toward malaoxon-inhibited mouse brain AChE under *in vitro* conditions (dos Santos et al., 2010). It is noteworthy that the reactivating effect of K074 was significantly higher when compared to that of pralidoxime when both oximes were present at equimolar concentrations. Based on these evidences, the present study aimed to investigate the *in vivo* reactivating effects of K074 toward AChE of malathion-poisoned mice. Its potential reactivating effect was compared with the commonly clinically used oxime (pralidoxime), whose reactivating efficacy has been questioned. Although several studies (Kassa et al., 2009) have explored the efficacy of protective agents against OP poisoning based on protocols with simultaneous exposures to both OP compounds and oximes, we performed an intoxication schedule where the oximes were administered 6 h after the OP (malathion) treatment. This was done in order to mimic a real condition where humans (mainly agricultures) spend several hours to reach emergency health services after an acute poisoning event. The ability of oximes in reactivating inhibited AChE was evaluated in the prefrontal cortex, hippocampus and blood of the poisoned animals. Considering the importance of oxidative stress in the malathion neurotoxicity, the effectiveness of K074 and pralidoxime to counteract this phenomenon was also evaluated in the prefrontal cortex and hippocampus.

2. Materials and methods

2.1. Chemicals

Commercial-grade malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithioate, 95% purity, CAS 121–75–5) was purchased from Dipil Chemical Ind. (Brazil). β-Nicotinamide adenine dinucleotide phosphate sodium salt reduced from 5,50-dithio-bis(2-nitrobenzoic) acid, glutathione reductase from baker's yeast and reduced glutathione were obtained from Sigma (St. Louis, MO, USA). Pralidoxime and K074 (see Fig. 1) of 98.5% purity were synthesized at the Department of Toxicology of the Faculty of Military Health Sciences in Hradec Kralove (Czech Republic). Their purity was analyzed using high performance liquid chromatography. All other chemicals were of the highest grade available commercially.

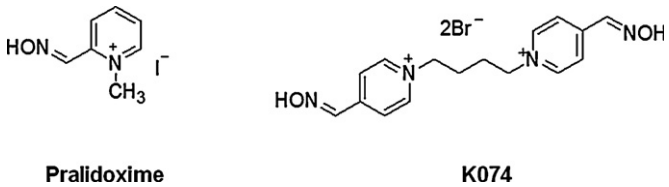
2.2. Animals

Adult Swiss male mice (60 days old), from our own breeding colony, were maintained at 22 °C, on a 12 h light:12 h dark cycle, with free access to food and water. All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology (1989) and were approved by our ethics committee for animal use at the Universidade Federal de Santa Catarina (PP00060/CEUA; 23080.024292/2006-22/UFSC).

2.3. Treatments

2.3.1. Intramuscular oximes injection

Forty-eight animals were randomly divided into eight groups (A–H) with 6 animals each. Animals from groups E–H received a single subcutaneous (s.c.) malathion injection (1.25 g/kg). Animals from groups D/F–H received a single intraperitoneal (i.p.) atropine sulfate injection (20 mg/kg, dissolved in saline). The oxime pralidoxime (66 mg/kg; 1/4 LD_{50}) or K074 (5.8 mg/kg; 1/4 LD_{50}), dissolved in saline, was administered intramuscularly (i.m.) to animals from groups B/G and C/H, respectively. LD_{50} were based on previous reports (Kassa and Humlicek, 2008; Kassa et al., 2005) and



Pralidoxime K074

Fig. 1. Chemical structure of oximes used.

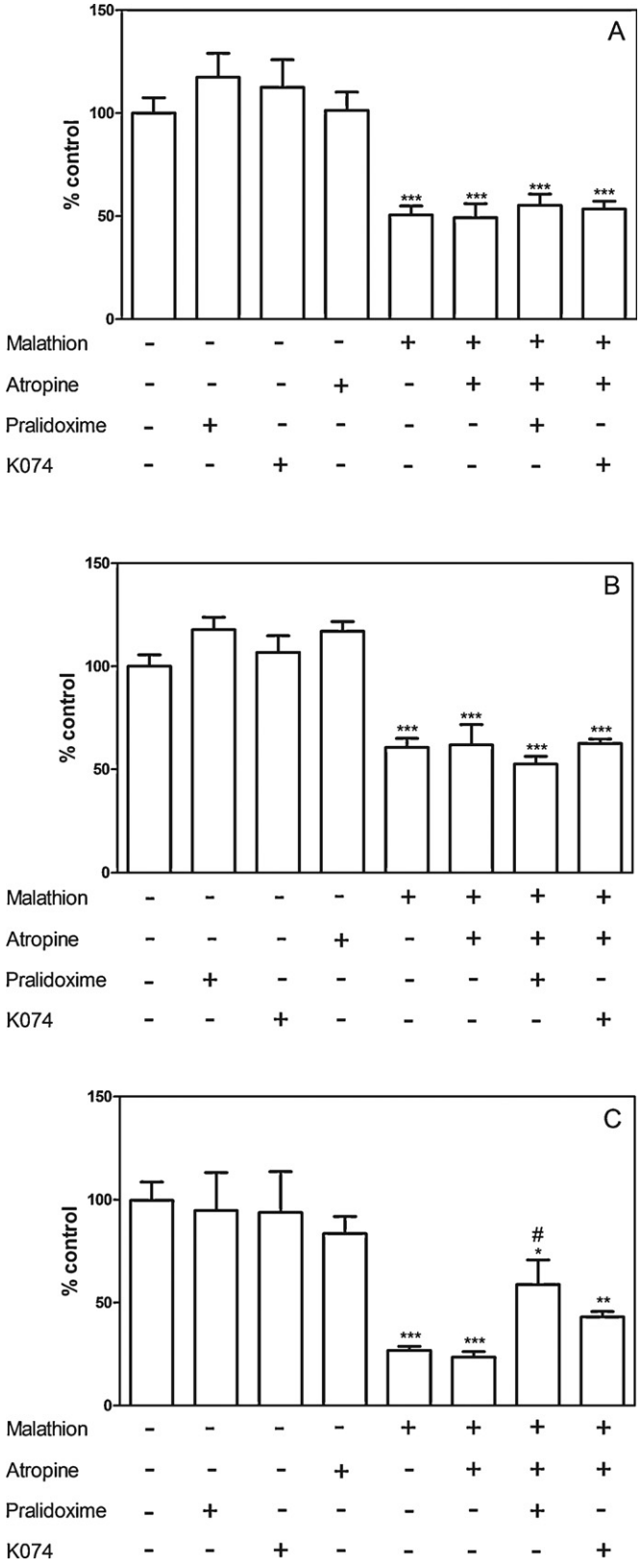


Fig. 2. Effects of malathion, atropine and oximes treatment on the AChE activity in the prefrontal cortex (A), hippocampus (B) and blood (C) of mice. Animals were treated with a single malathion injection (1.25 g/kg, s.c.) and 6 h after the malathion exposure the oximes were administered (1/4 LD_{50}, i.m.). Enzyme activity in the prefrontal cortex, hippocampus and blood is expressed as % of control (n = 6 animals per group). Basal AChE activity was 11.70 ± 2.533 nmol of substrate hydrolyzed/min/mg protein in the cortex, 5.374 ± 1.176 nmol of substrate hydrolyzed/min/mg protein in the hippocampus and 1.882 ± 0.7071 μmol of substrate hydrolyzed/min/mg Hb in the blood (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with the control treated with saline, $^{\#}p < 0.05$ when compared with malathion/atropine-treated group for the analysis of variance (ANOVA) followed by Duncan multiple range test.

on a preliminary toxicological study. Atropine (i.p.) and oximes (i.m.) were administered 6 h after malathion treatment. Malathion dose was based on a previous study from our group (da Silva et al., 2008) and this exposure protocol was developed in an attempt to mimic a real condition of severe human poisoning to malathion, characterized by high (around 70%) blood AChE inhibition (WHO, 1986). Vehicle (saline) administrations were performed in control the conditions. Animals from groups D/F–H received a single intraperitoneal (i.p.) atropine sulfate injection (20 mg/kg, dissolved in saline).

2.3.2. Intracerebroventricular oximes injection

Oximes (dissolved in saline) or saline was injected intracerebroventricularly (i.c.v.) 6 h after a single malathion injection (1.25 g/kg, s.c.). Briefly, a microsyringe with a specially made 28-gauge stainless steel needle, 3 mm in length, was used for microinjections. Mice were anaesthetized lightly with ether (just that necessary for loss of the postural reflex) and needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes (used as external reference). Pralidoxime (300 nmol/3 μl, ∼1/4 LD_{50}), K074 (30 nmol/3 μl, ∼1/4 LD_{50}) or saline was delivered gradually into the lateral ventricle, at the following coordinates from bregma: anteroposterior (AP) = −0.1 mm, mediolateral (ML) = 1 mm, and dorsoventral (DV) = −2.4 mm. LD_{50} were based on a preliminary toxicological study.

2.4. Tissue preparation for biochemical analyses

Twenty-four hours after malathion exposure, animals were submitted to ether anesthesia and the blood was collected by cardiac puncture in heparinized tubes. Then, the animals were killed by decapitation and the prefrontal cortices and hippocampus were removed and homogenized (1:10 w/v) in HEPES 25 mM, pH 7.4 buffer. The tissue homogenates were centrifuged at 3000 × g, at 4 °C for 5 min and an aliquot was used for the determination of AChE activity. Thereafter, the supernatants of the first centrifugation were again centrifuged at 16,000 × g, at 4 °C for 20 min and the supernatants obtained were used for the determination of enzymatic activities (glutathione peroxidase, glutathione reductase and catalase). For determination of blood AChE activity, whole blood was diluted (1:25, v:v) in ultrapure water and the hemolyzed sample was used for the measurement of AChE activity, which were normalized by hemoglobin (Hb) content. The Hb concentration was measured at 540 nm as cyano-met-Hb form based on Ventura et al. (1967) with minor modifications from Farina et al. (2002).

2.5. Biochemical analyses

2.5.1. Acetylcholinesterase activity

Brain and blood AChE activity was estimated by the method of Ellman et al. (1961), using acetylthiocholine iodide as a substrate. The rate of hydrolysis of acetylthiocholine iodide is measured at 412 nm through the release of the thiol compound which, when reacted with DTNB, produces the color-forming compound TNB.

2.5.2. Antioxidant enzymes

Glutathione peroxidase (GPx) activity was measured by the Wendel (1981) method, using tert-butylhydroperoxide as a substrate. NADPH disappearance was monitored by a spectrophotometer at 340 nm. Glutathione reductase (GR) activity was

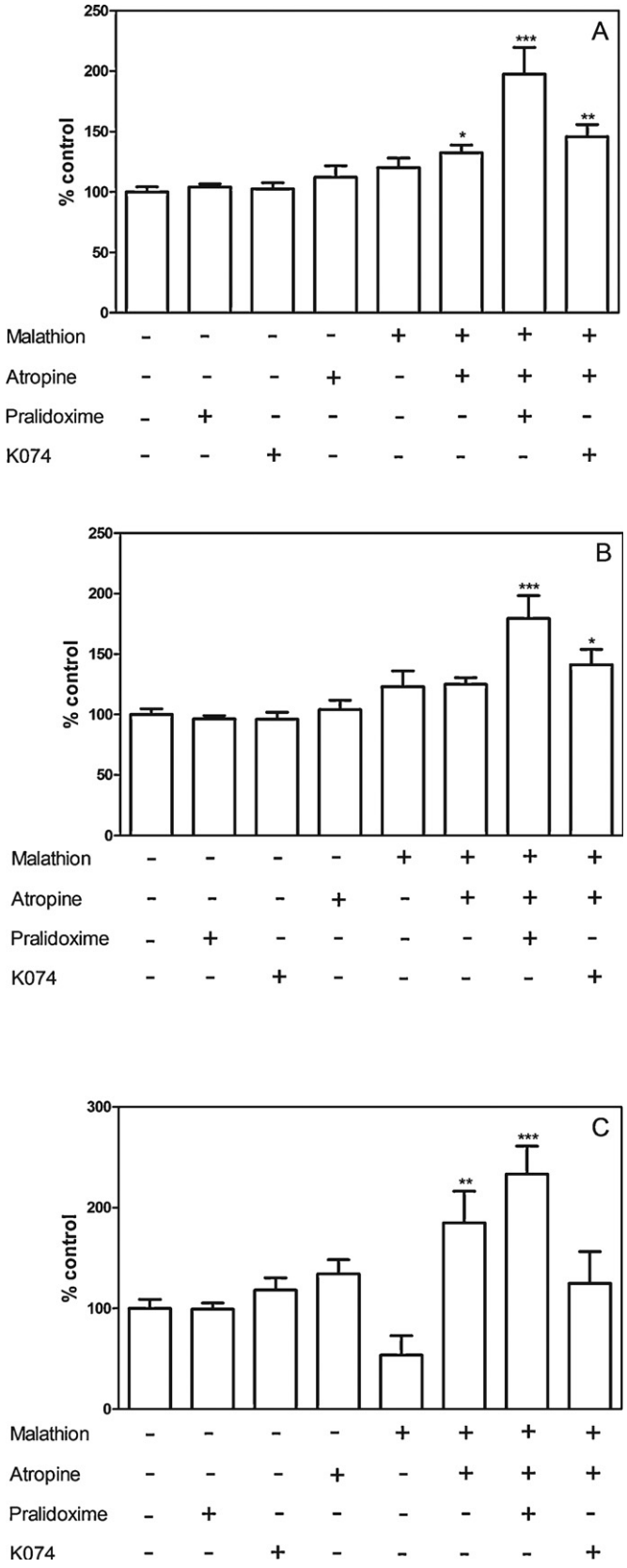


Fig. 3. Effects of malathion, atropine and oximes treatment on mouse prefrontal cortex glutathione reductase (A), glutathione peroxidase (B) and catalase (C) activities. Animals were treated with a single malathion injection (1.25 g/kg, s.c.) and 6 h after the malathion exposure the oximes were administered (1/4 LD_{50}, i.m.). Glutathione peroxidase (GPx), glutathione reductase (GR) and catalase activities are expressed as % of control ($n = 6$ animals per group). Basal enzyme activity was 34.05 ± 3.615 nmol of NADPH oxidized/min/mg protein for GR, 14.58 ± 0.7044 nmol of NADPH oxidized/min/mg protein for GPx and 0.7846 ± 0.1613 μmol of substrate hydrolyzed/min/mg protein for catalase (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with the control treated with saline by analysis of variance (ANOVA) followed by Duncan multiple range test.

determined by the method described by Carlberg and Mannervik (1985). The rate of GSSG reduction was indirectly determined through monitoring the NADPH disappearance at 340 nm. Catalase activity was measured by the method of Aebi (1974). The reaction was started by the addition of freshly prepared 30 mM H_2O_2. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm.

2.5.3. Determination of protein

The protein content was quantified by the method of Bradford (1976), using bovine serum albumin as a standard.

2.6. Statistical analysis

Differences between groups were evaluated by analysis of variance (ANOVA), followed by Duncan's multiple range tests when appropriate (statistical significance set at $p < 0.05$).

3. Results

First of all, we performed toxicological studies with either pralidoxime or K074 in order to find their LD_{50} in Swiss mice. Based on the mortality rate at 24 h after single i.m. or i.c.v. injections, we found LD_{50} values of approximately 264 and 23.2 mg/kg (i.m.) and 1200 and 120 nmol/site (i.c.v.) for pralidoxime and K074, respectively. Thereafter, we investigated the potential ability of these oximes (1/4 LD_{50}, i.m., in combination with atropine) in reversing malathion-induced AChE inhibition in the prefrontal cortex, hippocampus and blood of mice 24 h after malathion exposure (Fig. 2). It is important to emphasize that the potential antidotal treatments were administered 6 h after malathion treatment in order to mimic a real condition where humans (mainly agricultures) spend several hours to reach emergency health services after an acute poisoning event. One-way ANOVA showed significant effects of treatments on cortico-cerebral [$F(7, 41) = 12.97$; $P < 0.001$] (Fig. 2A), hippocampal [$F(7, 45) = 19.72$; $P < 0.001$] (Fig. 2B) and blood [$F(7, 42) = 8.86$; $P < 0.001$] (Fig. 2C) AChE activity. Malathion exposure significantly ($P < 0.001$) inhibited the AChE activity in the prefrontal cortex (around 50%), hippocampus (around 40%) and blood (around 70%). Pralidoxime or K074 (in combination with atropine) were unable to reverse malathion-induced AChE inhibition in the prefrontal cortex and hippocampus 18 h after oximes injection (Fig. 2A and B). Only pralidoxime (43%, $P < 0.05$) significantly reversed (although partially) the AChE inhibition induced by malathion poisoning in the blood (Fig. 2C).

Since oxidative stress has been proposed as an important mechanism involved with malathion-induced neurotoxicity, we investigated the effects of malathion and oximes exposures on biochemical parameters related to the antioxidant defenses (glutathione peroxidase, glutathione reductase and catalase) and thiobarbituric acid reactive substances (TBARS, marker of lipid peroxidation) in the prefrontal cortex and hippocampus (Figs. 3 and 4). This was performed in order to investigate whether the oximes (administered intramuscularly) could prevent the occurrence of malathion-induced changes on oxidative stress-related parameters in these encephalic structures, correlating these possible preventive effects with potential reactivating events. Fig. 3 shows the effects of malathion (s.c.) and oximes (i.m.) exposures on the activities of antioxidant enzymes from prefrontal cortex. The treatments with malathion, atropine, pralidoxime or K074 alone did not affect

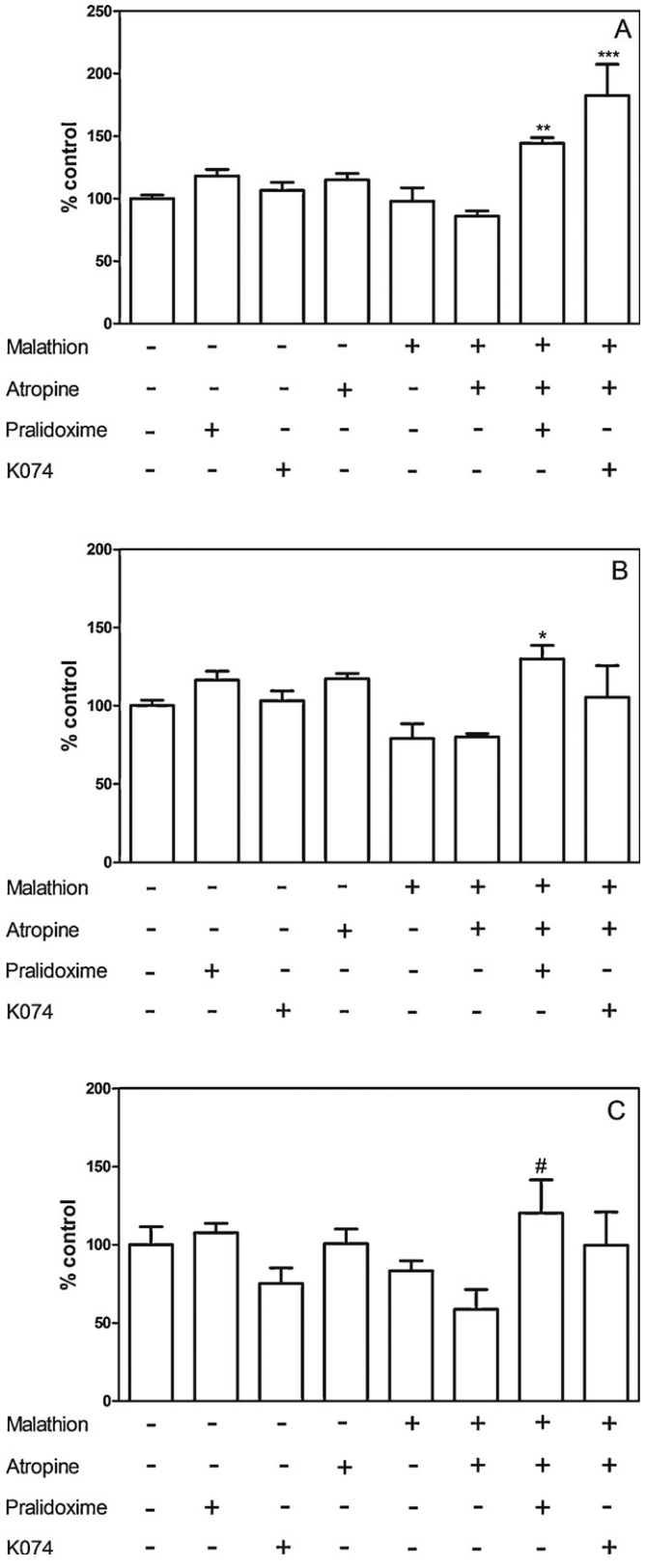


Fig. 4. Effects of malathion, atropine and oximes treatment on hippocampal glutathione reductase (A), glutathione peroxidase (B) and catalase (C) activities. Animals were treated with a single malathion injection (1.25 g/kg, s.c.) and 6 h after the malathion exposure the oximes were administered (1/4 LD_{50}, i.m.). Glutathione peroxidase (GPx), glutathione reductase (GR) and catalase activities are expressed as % of control (n = 6 animals per group). Basal enzyme activity was 38.29 ± 1.498 nmol of NADPH oxidized/min/mg protein for GR, 11.71 ± 0.9502 nmol of NADPH oxidized/min/mg protein for GPx and 0.7620 ± 0.1213 μmol of substrate hydrolyzed/min/mg protein for catalase (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with the control treated with saline, $^{\#}p < 0.05$ when compared with malathion/atropine-treated group by analysis of variance (ANOVA) followed by Duncan multiple range test.

glutathione peroxidase, glutathione reductase and catalase activities in the prefrontal cortex 24 h after administrations (Fig. 3). However, glutathione peroxidase ($P < 0.001$), glutathione reductase ($P < 0.001$) and catalase ($P < 0.001$) activities were significantly increased when pralidoxime was injected 6 h after the malathion poisoning, when compared with the control group. Moreover, the oxime K074 significantly increased glutathione peroxidase ($P < 0.05$) and glutathione reductase ($P < 0.01$) activities in the prefrontal cortex (Fig. 3A and B). Fig. 4 shows the effects of malathion and oximes exposure on the hippocampal activities of antioxidant enzymes. Similarly to the prefrontal cortex, the treatment with malathion, atropine, pralidoxime and K074 alone did not affect glutathione peroxidase, glutathione reductase and catalase activities in the hippocampus 24 h after treatments. However, both oximes significantly increased the glutathione reductase activity when injected 6 h after malathion poisoning [Fig. 4A; $P < 0.01$ (pralidoxime) and $P < 0.001$ (K074)]. Pralidoxime, but not K074, was able to increase glutathione peroxidase activity in the hippocampus (Fig. 4B; $P < 0.05$) when compared to the control group, as well as increase catalase activity in the hippocampus when compared to the malathion + atropine group (Fig. 4C; $P < 0.05$).

Although the intramuscular treatments with pralidoxime and K074 were able to increase the activities of antioxidant enzymes in the prefrontal cortex and hippocampus of malathion-treated animals, biochemical parameter related to the lipid peroxidation (TBARS) was not changed in these structures by treatments (data not shown).

Taking into account that the absence of significant reactivating effects of either pralidoxime or K074 toward hippocampal and cortical AChE (Fig. 2) could be due to the limited transport of both oximes through the blood–brain barrier (Bajgar et al., 2007; Lorke et al., 2007; Sakurada et al., 2003), a further experiment based on i.c.v. oxime administration was performed. As expected, malathion exposure significantly ($P < 0.01$) inhibited the AChE activity in the prefrontal cortex and hippocampus (around 50%) at 24 h after a single administration. The i.c.v. injections of pralidoxime or K074, which were performed at 6 h after malathion poisoning, were unable to reverse malathion-induced AChE inhibition in the prefrontal cortex and hippocampus (Fig. 5A and B).

4. Discussion

Organophosphorus (OP) compounds are highly toxic agents that represent the largest class of insecticides sold worldwide. They are widely and effectively used in agriculture, and to a lesser extent, in domestic pest control (Jaga and Dharmani, 2003; Kamanyire and Karalliedde, 2004). Particularly important, data published by our Toxicological Information Center shows that malathion, a well-known OP compound, is featured on the record of human intoxications in Santa Catarina, a state in Southern Brazil.

The toxic effects elicited by acute malathion poisoning are mainly attributed to the inhibition of AChE in the nervous tissue, leading to increased acetylcholine levels in the synaptic cleft and causing cholinergic syndrome (Kwong, 2002). The standard clinical treatment for malathion poisoning, which represents the combination of anticholinergic drugs (atropine) and AChE reactivators (oximes), is not sufficiently effective (Sudakin et al., 2000). Of particular importance, the lack of brain penetration is a major limitation for the currently used oximes as antidotes of OP poisoning (Kalisiak et al., 2011). Accordingly, pralidoxime, a widely used oxime in OP poisoning, seems to be a poor reactivator of OP-inhibited AChE (Kuca et al., 2010) and there are biochemical

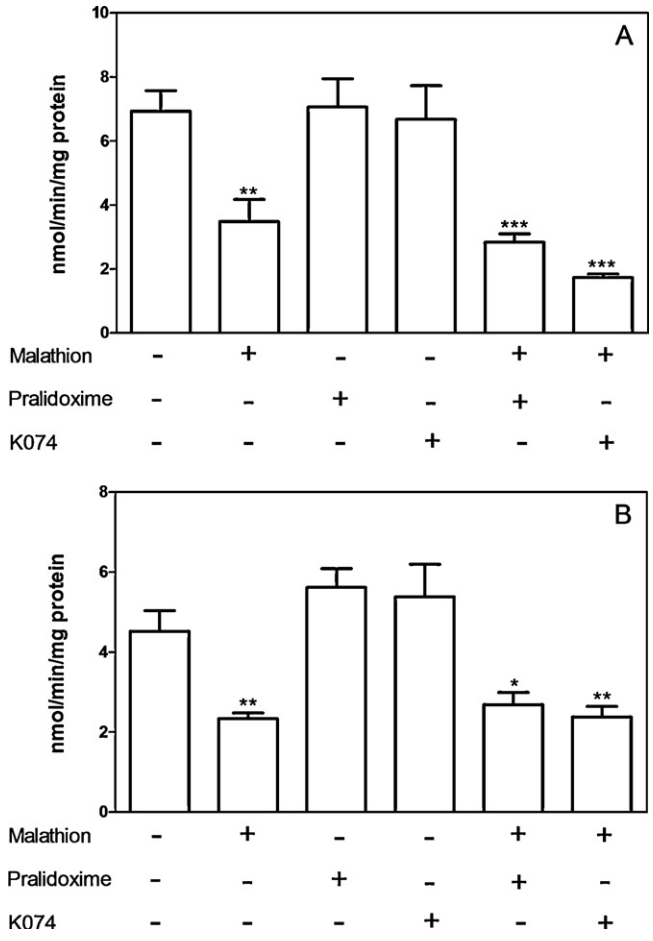


Fig. 5. Effects of malathion and oximes treatment on the AChE activity in the prefrontal cortex (A) and hippocampus (B) of mice. Animals were treated with a single malathion injection (1.25 g/kg, s.c.) and 6 h after the malathion exposure the oximes were administered (1/4 LD_{50}, i.c.v.). Enzyme activity in the prefrontal cortex and hippocampus is expressed as nmol of substrate hydrolyzed/min/mg protein and represented as mean ± SEM ($n = 5$ animals per group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with the control treated with saline for the analysis of variance (ANOVA) followed by Duncan multiple range test.

and clinical evidences that it does not reactivate human AChE inhibited by malathion exposure (Ganendran and Balabaskaran, 1976). Nevertheless, pralidoxime has been commercially available for many years and it is still considered as a gold standard of AChE reactivators (Kuca et al., 2010). From a clinical point of view, there is a clear demand for more effective reactivators of malathion-inhibited AChE to efficiently alleviate the neurotoxic symptoms.

Previous studies from our group have demonstrated that oximes from the K-series (developed by Kuca from Department of Toxicology of the Faculty of Military Health Sciences, Hradec Kralove, Czech Republic) namely K027, K048, K074 and K075 are potent reactivators of AChE inhibited by malathion/malaoxon under *in vivo* and *in vitro* conditions (da Silva et al., 2008; dos Santos et al., 2010). Our previous *in vitro* study showed that the oxime K074 presented an outstanding reactivating effect toward malaoxon-inhibited mouse brain AChE, which was significantly higher when compared to that of pralidoxime when both oximes were present at equimolar concentrations (dos Santos et al., 2010). However, in this *in vivo* study, efficacy comparisons using equimolar doses of the tested oximes were not performed because of their different *in vivo* toxicities. Based on literature data and on previous toxicity studies developed in our laboratory, we observed that the toxicities of standard oximes studied in rodents are HI-6 < pralidoxime < obidoxime < trimedoxime (Calic et al., 2006; Musilek et al., 2007). Regarding to the oximes from K-series, their toxicities are K027 < K048 < K075 < K074 (Musilek et al., 2007). Taking this into consideration, in this study we used 1/4 LD_{50} for both oximes and the results showed that pralidoxime and K074 (intramuscularly administered) were not able to reactivate the inhibited AChE in prefrontal cortex and hippocampus of mice at least within the first 24 h after malathion exposure. In contrast, pralidoxime, but not K074, significantly reversed (although partially) the AChE inhibition induced by malathion poisoning in the whole blood. These results are in agreement with recent studies in guinea pigs where oximes (e.g. pralidoxime) exhibited differential potency in reactivating nerve agent-inhibited cholinesterases (ChE) in various peripheral tissues and blood, but did not affect ChE activity in brain regions (Shih et al., 2009, 2010). These data are particularly important taking into consideration the use of blood AChE as an important parameter to predict the inhibition status of the synaptic enzyme (Eyer et al., 2007). The data suggest that peripheral and central AChE activities are not necessarily correlated after the treatment of OP compounds and/or oximes, which should be taken into account in the diagnosis and management of OP-exposed humans.

The effectiveness of oxime-mediated reactivation is primarily attributed to the nucleophilic displacement rate of organophosphates, but the reactivating efficiency varies with the chemical structure of the OP compound, the source of enzyme, the chemical structure of the oxime and the rate of postinhibitory dealkylation, known as aging (Kovarik et al., 2004; Worek et al., 2002). In the present study, the reactivating effects of pralidoxime were relatively low when compared to their high reactivating effects toward malaoxon-inhibited AChE under *in vitro* conditions (dos Santos et al., 2010). In fact, the results of the present study showed no significant reactivating effects of both oximes toward cortico-cerebral and hippocampus AChE. Moreover, a modest (although significant) reactivating effect of pralidoxime was observed toward blood AChE. These events should be related, at least in part, to the rate of postinhibitory dealkylation (aging), which may be a consequence of the exposure schedule. In fact, the administration of the oximes at 6 h after malathion poisoning likely allows for the occurrence of high levels of dealkylation in the enzyme–inhibitor complex, decreasing the probability of reactivation by oximes. This experimental evidence is in agreement with the lack of satisfactory reactivating effects of oximes in malathion poisoning, suggesting that the rate of AChE aging in malathion poisoning is high. From a clinical point of view, it is important to emphasize that our exposure protocol was performed in an attempt to mimic real conditions, in which humans (mainly rural workers) poisoned by malathion do not achieve immediate health care after acute intoxication.

Kinetic parameters certainly may affect the inhibitory efficacy of OP compounds, as well as the reactivating effects of oximes. In this regard, high bioavailabilities of OP compounds and oximes and their distributions through the blood–brain barrier (BBB) should be crucial for the reactivation of the brain enzyme. The transport of pralidoxime, as well as other positively charged hydrophilic small oximes (obidoxime, K027 and K048) through the BBB is only minimal (Bajgar et al., 2007; Lorke et al., 2007; Sakurada et al., 2003). Sakurada et al. (2003) have reported that the mean BBB penetration of pralidoxime is approximately 10%. In addition, it was suggested that the ratio of brain extracellular fluid to blood concentration of pralidoxime would be lower after OP poisoning because pralidoxime is used for reactivation of inhibited AChE in blood prior to BBB penetration (Sakurada et al., 2003). It is important to emphasize here that the ability of the oxime K074 in crossing the BBB has not been well described in literature. However, in this study, the absence of reactivating effects toward brain AChE suggests that K074 may have limited access to the CNS.

Taking into account the possibility that these oximes do not effectively cross the BBB, a protocol of i.c.v. administrations was performed. However, even after i.c.v. injection, pralidoxime or K074 were unable to reverse malathion-induced AChE inhibition in the prefrontal cortex and hippocampus. These results indicate that both oximes, which were intracerebroventricularly injected at high levels (1/4 LD_{50}), were unable to destabilize the complex formed between hippocampal/cortical AChE and malaoxon, at least when administered 6 h after malathion exposure. The data suggest that the rate of postinhibitory dealkylation (aging) represents a crucial event in the CNS, which should be taken into account when using oximes to treat malathion poisoning. In agreement with this idea, our data showed that i.m. pralidoxime administration significantly reversed malathion-inhibited blood AChE, but i.c.v. pralidoxime administration did not change the activity of malathion-inhibited hippocampal and cortical AChE activity, suggesting that *AChE aging* seems to depend on the tissue source (blood vs. CNS). Although literature data on this theme are scarce, Sket and Brzin (1986) investigated the rate of aging of soman-inhibited brain AChE and showed a statistically significant reactivation of inhibited AChE in brain only when the oxime (i.c.v. injected) was applied up to 20 min after soman. Then, it is possible that an effective reactivating effect with pralidoxime and K074 could be achieved in our model of malathion poisoning if these oximes were administered immediately after the malathion exposure. However, additional kinetic studies are necessary to investigate this hypothesis.

There is evidence for an important role of oxidative stress in the toxicity elicited by OP agents. Recent studies have shown that malathion exposure induces oxidative stress in different animal models by increased lipid peroxidation levels (TBARS) in mouse and rat brain (Brocardo et al., 2007; da Silva et al., 2008), altered activities of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase and catalase (Fortunato et al., 2006), as well as DNA damage in brain structures after short-term (Brocardo et al., 2007) and chronic treatment with malathion (Reus et al., 2008). However, the exact mechanism involved in this phenomenon is not fully understood. In our experiments, no changes were observed in enzymatic antioxidant defenses (GR, GPx and catalase activities) and TBARS levels in prefrontal cortex and hippocampus 24 h after the malathion exposure. Other results are in agreement with these data, such as those reported by Acker et al. (2009), which have shown that the exposure to malathion in rats did not alter parameters of oxidative stress, such as TBARS levels and non-enzymatic and enzymatic antioxidant defenses.

Despite the oximes are compounds generally used to reverse the AChE inhibition caused by OPs (Worek et al., 2004), studies have proposed that cholinesterase reactivators with antioxidant properties may be a good approach for OP intoxication treatments (Puntel et al., 2008, 2009). In a previous study from our group, we showed that the oximes from K-series, K027 and K048 were able to reduce the lipid peroxidation in the prefrontal cortex of mice (da Silva et al., 2008). Additionally, some oximes were able to decrease TBARS production in brain homogenates induced by malonate and iron (Puntel et al., 2008) and also exhibited a protective role during *in vitro* Cu^{2+}-induced LDL and serum oxidation (de Lima Portella et al., 2008). In earlier experiments, we measured the hydroxyl scavenging activity of the pralidoxime and K074 using an *in vitro* $DPPH^{\bullet}$ assay. The direct interaction between oxime and radical $DPPH^{\bullet}$ was analyzed and the results showed that pralidoxime and K074 were not able to act against the reactive species $DPPH^{\bullet}$, showing no antioxidant activity in this assay. Interestingly, in the present study, the treatments with pralidoxime and K074 alone were not able to increase the endogenous activities of antioxidant enzymes in the prefrontal cortex and hippocampus 18 h after oximes injection. However, the activity of these enzymes was significantly increased when the oximes were administered after malathion poisoning. From a molecular point of view, it is difficult clearly to explain these results only based in our results. In addition, studies about this theme are absent. Thus, additional studies need to be performed in an attempt to elucidate this phenomenon.

In conclusion, the present study showed that the oxime K074 and the commonly used oxime pralidoxime were not able to reactivate inhibited AChE in mouse prefrontal cortex and hippocampus when administered 6 h after malathion exposure. However, in the blood, pralidoxime significantly reversed the AChE inhibition induced by malathion poisoning. Moreover, i.c.v. administration of both oximes (6 h after malathion poisoning) did not change the inhibitory effect elicited by this OP compound on hippocampal and cortical AChE activity. Altogether, the results indicate that: (i) peripheral (blood) and central (hippocampal and cerebro-cortical) AChE activities are not necessarily correlated after OP exposure and/or oxime treatment; (ii) the rate of postinhibitory dealkylation (aging) represents a crucial event in the CNS, which seems to be kinetically different when compared to peripheral tissues. These events should be taken into account in the diagnosis and management of OP-exposed humans. In addition, because AChE is a key target concerning the toxic effects elicited by OP compounds and considering that the available treatments (based on oximes, especially pralidoxime) to malathion poisoning appear to be ineffective, the present study reinforce the need to search for potential new AChE reactivators able to efficiently reactivate the brain and blood AChEs after malathion poisoning.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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